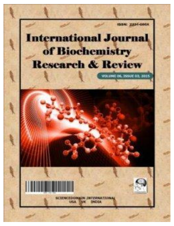

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The Response of Antioxidant Enzymes and Photosynthesis Dynamics of Sunflower Exposed to Aniline Wastewater

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Authors' contributions

This work was carried out in collaboration between all authors. Author TJ designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author HCX managed the analyses of the study and wrote the final draft. Author MC managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: In order to investigate the tolerance of sunflower to aniline, we measured the photosynthetic parameters, chlorophyll fluorescence parameters and antioxidant enzyme activities of sunflower in aniline wastewater.
Study Design: The experiment was conducted with oil sunflower No. 4 (*Helianthus annuus Linn*) as the experimental material. After five weeks of culture in half strength Hoagland's nutrient solution, seedlings with uniform growth status were selected for aniline stress treatment. The concentrations of aniline stress treatment were 0, 60, 80, 100, 120, 140, 160 and 180 mg/L, respectively. Each treatment has five replicates. Leaf photosynthetic parameters were measured at fifth and tenth day after treatment under natural environmental conditions.
Place and Duration of Study: Place for the study was located at Shandong Agricultural University, Taian city, Shandong, China. The experiment lasted for 10 weeks.
Results: Five days after stress treatment, the net photosynthesis rate (P_n), stomatal conductance

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(G_s), and transpiration rate (T_r) of sunflower showed an upward trend at the lower concentration of aniline (≦100 mg/L) and turned to a downward trend at higher concentration of aniline (≥120 mg/L). However, ten days after stress treatment, P_n, G_s and T_r of sunflower of all concentrations decreased significantly (P=.05) as compared to the control. The photochemical quenching coefficient and effective quantum yield of PS II photochemistry decreased. At the end of the ten days experiment, with the increase of aniline concentration, the photosynthetic and chlorophyll fluorescence parameters of sunflower showed a downward trend except for intercellular CO_2 concentration. The data indicated that nonstomatal limitation was responsible for the reduction of P_n. When the aniline concentration reached 180 mg/L, the activity of superoxide dismutase (SOD) and peroxidase (POD) reached the highest point, which was 3.49 times and 1.78 times higher than those of the control.
Conclusion: The low concentration of aniline (<120 mg/L) could promote the normal growth of sunflower in a short time. However, when the stress duration reaches a certain level, aniline will have a significant toxic effect on sunflower, thereby severely affecting the normal growth of sunflower.

Keywords: Aniline pollution; tolerance; chlorophyll fluorescence; SOD; POD.

1. INTRODUCTION

Aniline is the most representative substance of aromatic amines. It is one of the main pollutants in the printing and dyeing industry, pharmaceutical industry, agriculture and other industries in China. With the development of these industries, the emission of aniline in the environment increases every year. Aniline's toxicity is strong and can inhibit the growth of aquatic animals and plants. Aniline enters the targets mainly through the skin, the respiratory tract, and the digestive tract. It can cause hemolytic anemia and toxic hepatitis. For these reasons, China has included aniline in the blacklist of China's environmental priority pollutants, and the United States EPA also ranked aniline as one of the 129 priority pollutants [1,2].

Compared with the commonly used physical, chemical and microbial remediation methods, phytoremediation can not only remove pollutants but also promote the recycling and reuse of nutrients in sewage. It can beautify the environment, mitigate regional climate and promote ecosystem well cycling [3]. Liu [4] used the wheat grass (*Agropyron cristatum (L.) Gaertn.*) to carry on the plant restoration experiment to the aniline contaminated soil, and discovered that the ice grass root system could absorb the aniline, and then transported it to leaf and panicle through the stem. Wang and Wen [5] found that the efficiency of phytoremediation of aniline in water was 50.7%~97.3% and water spinach *(Ipomoea aquatica Forsk)* had the best purification effect.

Sunflower (*Helianthus annuus*), a species in genus *Helianthus* under Asteraceae family, is a kind of salt resistance, drought resistance and barren adaptability oil crops [6-8]. Zhao [9] compared the remediation effects of soil uranium pollution on 5 plants, found that sunflower and sauteed green beans (*Phaseolus vulgaris L*) were effective plants for phytoremediation of soil polluted by uranium sequestration. Some sites in the United States used sunflowers to repair radionuclides contaminated groundwater [10]. Xie [11] found that sunflower had different tolerance and percent of decolorization to different kinds of azo dye wastewater. All the previous studies revealed that sunflower has good environmental adaptability, foundation and potential in the purification of Aniline Wastewater. However, the photosynthetic physiological responses of sunflower to aniline wastewater have not been reported yet. In this study, oil sunflower No. 4 was used to analyze the effects of different concentrations of aniline stress on photosynthesis, chlorophyll fluorescence parameters and antioxidant enzyme activities of sunflower. This study aimed to reveal the tolerance and adaptation mechanism of sunflower to aniline stress and to provide a fundamental scientific understanding of phytoremediation of aniline in water.

2. MATERIALS AND METHODS

2.1 Experimental Design

- The present study was conducted with oil sunflower No. 4 (*Helianthus annuus Linn.*) as the experimental material. The plump seeds with the same size were selected.

After 24 hours soaking in distilled water, the seeds were sowed in sterilized quartz sand, and watered with deionized water. Germination of the seeds was under natural conditions. Healthy seedlings were selected and placed in 250 mL volumetric flasks which were put on the window sill with a sunny exposure. After 5 weeks of culture in half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950), seedlings with uniform growth status were selected for aniline stress treatment.

The aniline dissolved in half strength Hoagland's nutrient solution as simulated wastewater. Aniline concentrations of stress treatment were 0 (control), 60, 80, 100, 120, 140, 160 and 180 mg/L, respectively. Each treatment has 5 replicates. Leaf photosynthetic parameters were measured under natural environmental conditions at 5^{th} and 10^{th} day after treatment.

2.2 Items and Methods of Measurement

2.2.1 Measurement of photosynthetic physiological parameters

Under the shiny weather condition, three mature leaves were selected for the determination of photosynthetic parameters from the middle part of each seedling. The net photosynthetic rate (P_n), transpiration rate (T_r), stomatal conductance (G_s) and the intercellular CO_2 concentration (C_i) were measured by the CIRAS-2 photosynthesis system produced by the British PPS company. For CO_2 assimilation parameters, 10 repetition data were acquired per treatment. Water use efficiency (WUE) and stomatal limitation (L_s) were calculated by the following equations:

$WUE=P_n/T_r$ [12], $L_s=1-C_i/C_a$ [13]

Where C_a is atmospheric CO_2 concentration.

2.2.2 Chlorophyll fluorescence parameter determination

After 30 min activation in natural light, minimal fluorescence yield of the light-adapted state (F_o'), maximal fluorescence yield of the light-adapted state,(F_m') and steady-state fluorescence yield, (F_s) of sunflower leaves under different aniline concentration gradients were measured using a pulsed modulated fluorescence system (FMS2.02 type, Hansatech, UK). The minimal fluorescence (F_o) and the maximal fluorescence (F_m were measured after 30 min dark adaptation. For chlorophyll fluorescence parameters, 10 repetition data were acquired per treatment. Parameters of optimal/maximal photochemical efficiency of PS II in the dark were calculated by the following equation:

$F_v/F_m=(F_m-F_o)/F_m$ [14]

Parameters of the actual photochemical efficiency of PS II in the light were calculated by the equation as follows:

$\Phi_{PS\,II}=(F_m'-F_s)/F_m'$ [14]

Photochemical quenching was calculated by the following equation:

$qP=(F_m'-F_s)/(F_m'-F_o')$ [15]

Nonphotochemical quenching was calculated by the following equation:

$NPQ=(F_m-F_m')\ /F_m'$ [15]

2.2.3 Enzyme activity assay

At the end of the experiment, sunflower leaves were taken, washed with distilled water, and dried with absorbent paper, and the enzyme activity of the leaves was measured. Determination of SOD and POD activity was performed by the method of Liu Ping [16]. For SOD and POD, 5 repetition data were collected per treatment.

2.3 Data Analysis

SPSS 17.0 and Excel 2010 software were used for data analysis and mapping. The difference between control and treatment was analyzed by one-way analysis of variance (ANOVA) and Duncan's multiple-range test was used for multiple comparisons.

3. RESULTS

3.1 Effects of Aniline Stress on Sunflower Photosynthesis

The change of photosynthetic parameters of sunflower, after exposed to aniline for 5 days, indicated that in the fifth day, the net photosynthetic rate (Pn) of sunflower leaves increased at lower aniline concentration (≦100 mg/L) and decreased at higher aniline concentration (≧120 g/L) (Fig. 1). While aniline concentration was 100 mg/L, the Pn was the highest (28.77 μmol CO_2·m-$2s^{-1}$), 1.28 times as high as that of the control group (P=.05). When the aniline concentration reached 140 mg/L, the Pn of sunflower decreased significantly (*P*=.05). When the aniline concentration reached 180 mg/L, P_n was only 66.88% of the same measurement from the control group. The results showed that low concentration of aniline promoted the photosynthesis of sunflower, and high concentration of aniline inhibited the photosynthesis. The inhibitory effect increased with the increase of aniline concentration.

With the increase of aniline concentration, the stomatal conductance (Gs), transpiration rate (Tr) and stomatal limitation value (Ls) changed with the same trend as Pn's response to the treatment, i.e., increasing at the initial stage and then decreasing. The changing trend of Ci was opposite to that of Pn. When the concentration of aniline was greater than 100 mg/L, the increase of aniline concentration resulted in a significantly reduced Ls, but increased Ci (*P=.05*). Those results showed that main reason for the decline of Pn was the nonstomatal factor under higher anline concentration (≧120 g/L).

When the experiment was carried out for ten days, along with the increase of aniline concentration, the P_n, G_s, L_s, T_r and *WUE* of sunflower showed a monotone downward trend, while C_i showed an upward trend. When the concentration of aniline was 60 mg/L, the

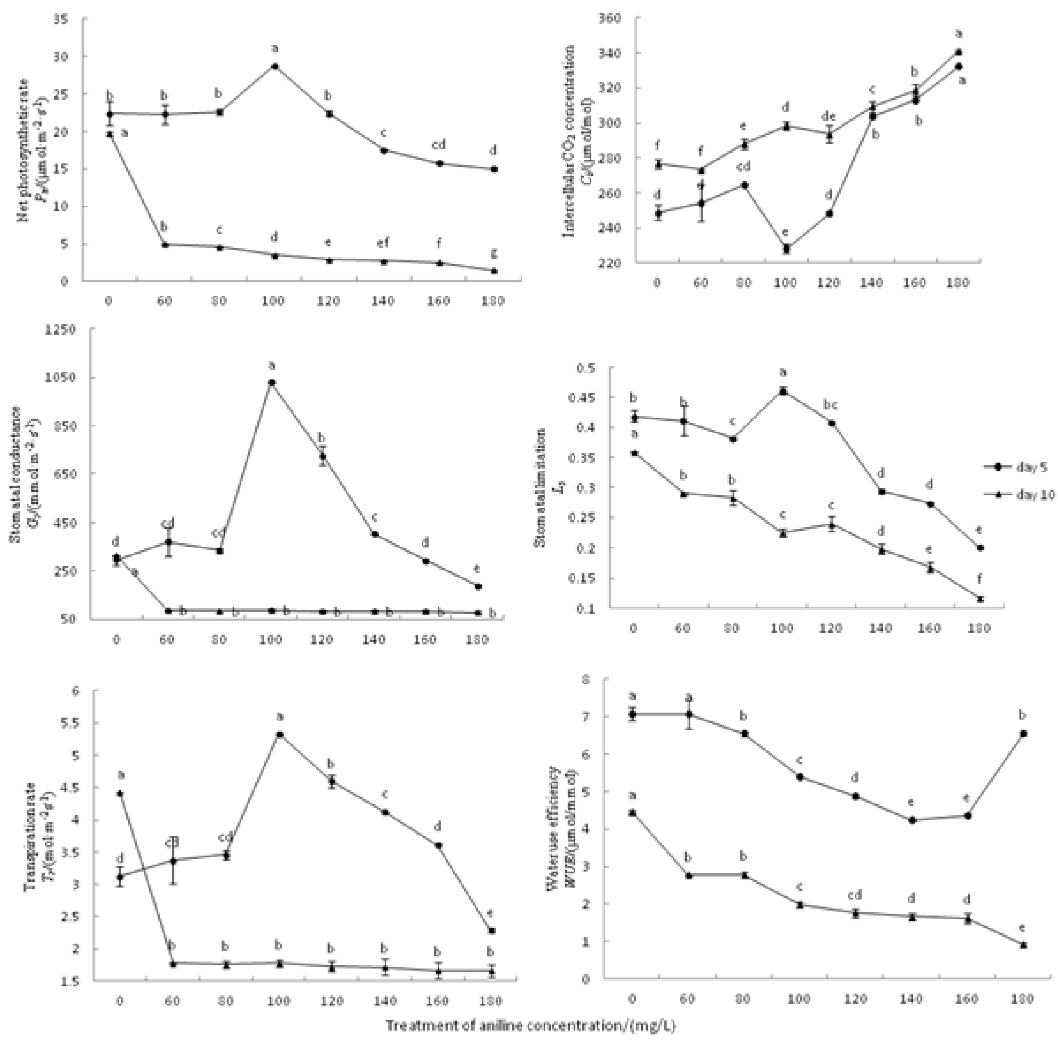

Fig. 1. Photosynthetic parameters of sunflowers under aniline stress

Values represent Mean ± S.E.M = Mean values ± Standard error of means, n=10. Different lower case letters indicate that means are significantly different from each other (p<0.05)

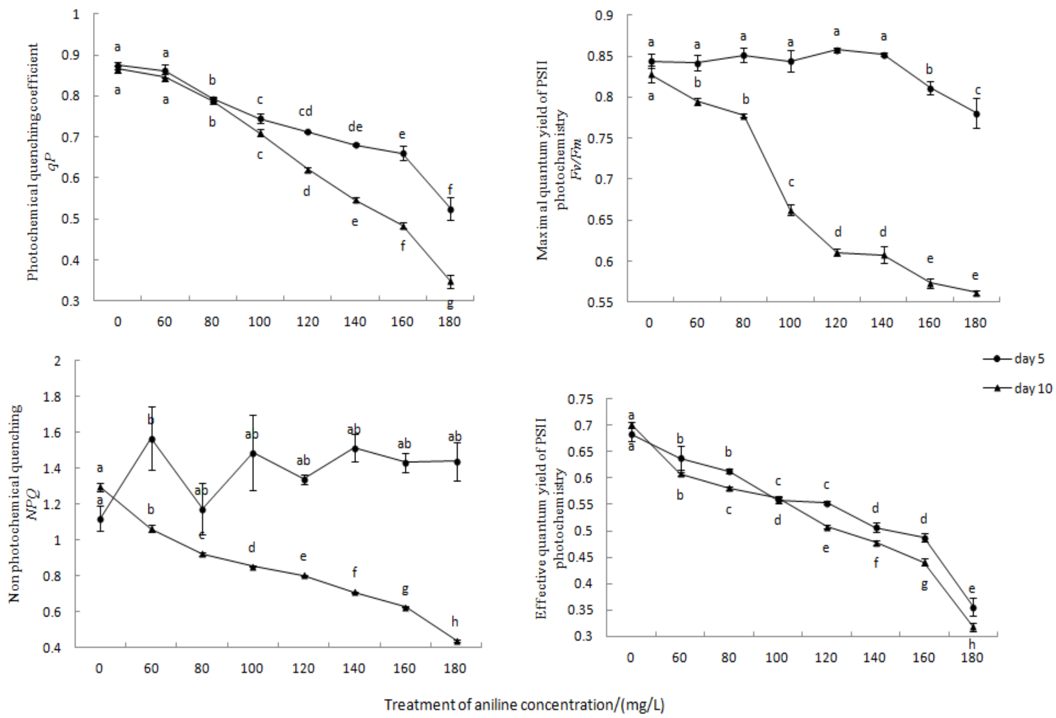

Fig. 2. Fluorescence parameters of sunflowers under aniline stress

Values represent Mean ± S.E.M = Mean values ± Standard error of means, n=10. Different lower case letters indicate that means are significantly different from each other (p<0.05)

P_n, G_s, L_s, T_r and *WUE* in the treatment group were significantly lower than those in the control group (*P*=.05), while the trend of C_i was contrary to that of P_n. Compared with the fifth day test, the P_n, G_s, L_s, T_r and *WUE* in the experimental group were significantly decreased. When aniline concentration was 100 mg/L, the reduction of Pn reached the lowest value, and it was 12.28% of the value on the fifth day.

3.2 Effect of Aniline Stress on Fluorescence of Sunflower

In the fifth day after aniline exposure, along with the increase of the concentration of aniline, $\Phi_{\text{PS II}}$ and *qP* were significantly decreased and the maximum chemical efficiency (F_v/F_m) of PS II decreased significantly when the concentration of aniline was 160 mg/L. The non photochemical quenching coefficient (*NPQ*) was significantly higher than that of the control group only when the concentration of aniline was 60 mg/L (*P*=.05). When the concentration of aniline was greater than 60 mg/L, there was no significant difference between the treatment group and the control group (*P*>.05). In the tenth day, along with the increase of aniline concentration, *qP*, PS II, F_v/F_m and *NPQ* were significantly lower than the control group (*P*=.05). The results showed that aniline stress could obviously inhibit or decrease the photochemical activity and photochemical efficiency of PS II. The longer the stress lasted, the more serious the stress effect would be.

3.3 Effect of Aniline Stress on SOD and POD

The activity of protective enzymes SOD and POD is closely related to the resistance of plants to stress. Along with the increase of aniline concentration, the SOD and POD activity of sunflower were more active (Fig. 3). When aniline concentration reached 180 mg/L, the activity of SOD and POD reached the highest point, 3.49 times and 1.78 times of the concentration from the control.

4. DISCUSSION AND CONCLUSION

Photosynthesis is the basis of plant growth, while stress has more obvious effects on photosynthetic physiology of plants. The reduction in the rate of photosynthesis indicates

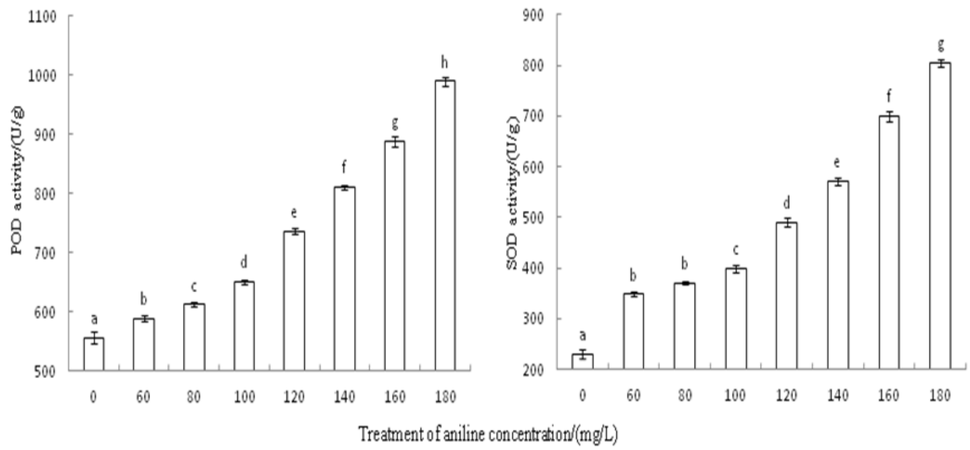

Fig. 3. Activity of SOD and POD of sunflowers under aniline stress

Values represent Mean ± S.E.M = Mean values ± Standard error of means, n=5. Different lower case letters indicate that means are significantly different from each other (p<0.05)

that the growth of a plant is depressed by the stress of a pollutant or other adverse factors [17]. Zhang [18] found that low temperature, weak light and salt stress could reduce net photosynthesis of pepper, and the inhibition effect of compound stress was greater than that of single stress. Generally, the factors that affect plant photosynthesis include stomatal factors and non-stomatal factors. The stomatal factors include water stress leading to stomatal conductance decline, CO_2 into the leaves blocked, and photosynthesis decline. The non-stomatal factors refer to the decreasing photosynthetic activity of mesophyll cells [19]. This study showed (Fig. 1) when the test with the concentration of aniline at 100 mg/L, was carried out to the fifth day, the net photosynthetic rate of sunflower was significantly higher than that of the control (*P*=.05). It can be suggested that low concentration of aniline could promote the physiological activities of sunflower. When the aniline concentration reached or exceeded 140 mg/L, aniline obviously inhibited the photosynthesis of sunflower. It can be said that greater the concentration, the stronger the inhibition ability. The decline in *Pn* was accompanied by a rise in *Ci* and a fall in *Ls*, the main limiting factor for photosynthesis at this time was non-stomatal factors [20], which damaged the photosynthetic apparatus of sunflower. Li Hui [21] found that while concentration of phenol is relatively low (50 mg/L), net photosynthesis of salix seedlings decreased. Xie [11] showed that the net photosynthetic rate of sunflower in three kinds of azo dyes (Bismarck brown, Evans blue, orange) at a concentration of 100 mg/L decreased significantly. Wang's [22] study showed that the low net concentration of naphthalene (10 mg/kg) increased the net photosynthetic rate, but high concentrations were markedly inhibited photosynthesis activity. However, the same concentration of 1,2,4-trichlorobenzene could significantly inhibit the net photosynthetic rate of rice. All the above studies show that the toxicity of organic matter to plants was very different. The photosynthetic physiological response of the plant to organic pollution stress also showed the difference. When the aniline stress time reached 10 days, the multiple photosynthetic indexes of sunflowers (*Pn*, *Tr*, *Gs*, *Ls*, *WUE*) were significantly lower than those in the control group (*P*=.05). The gap between test indexes on the fifth day was relatively large. The normal physiological activity of sunflowers could be seriously affected even if the aniline stress was prolonged, and even if the concentration of aniline was not high (60 mg/L).

Chlorophyll fluorescence parameters play a unique role in determining the absorption, transmission, dissipation and distribution of light energy during photosynthesis of plants [20]. *Fv/Fm* is PS Ⅱ maximum quantum yield of the photochemical reaction, reflects the PS Ⅱ reaction center of light energy conversion efficiency [23]. The parameter change found by this study was very small under the condition of non-stress, not affected by species and growth condition, and the parameter significantly decreased under stress. By the fifth day of the anline exposure, when the concentration of aniline was less than 160 mg/L, the data in the treatment group and the control group were not different significantly (Fig. 2), this suggested that in a short period of time, low concentration of aniline stress did not seriously affect the sunflower PS II system. FPS Ⅱ affected the proportion of light energy that was absorbed by the photosynthetic apparatus for photochemical reactions in the sun [24]. *QP* indicated that how the excitation energy used by PS II reaction center, the higher the value, the higher the electron transfer activity of PS II [25]. This experiment showed that (Fig. 2), as the concentration of aniline increased, *QP* and FPS Ⅱ all showed a trend of decline. It meant that aniline stress increased the PS Ⅱ reaction center closure degree and slowed the photosynthetic electron transport rate. The light energy for PS Ⅱ actual chemical reaction has been reduced [24]. As compared to the control group, with the increase of aniline concentration, *NPQ* showed a wave trend, and it increased significantly only when the concentration of aniline was 60 mg/L (*P*=.05). This indicated that the sunflower's light damage defense mechanism was activated, the light energy absorbed by PS Ⅱ of which the amount of heat dissipation which use by antenna pigment increased, thus helped ease the destruction of PS Ⅱ reaction center institutions caused by aniline stress [26]. In the tenth day after the aniline exposure, the *Fv/Fm* decreased significantly when the concentration of aniline was 60mg/L, which reflected that there was photoinhibition in the photosynthesis of sunflower leaves or the PS Ⅱ system was destroyed. The decline of Fv/Fm and PS II showed a decrease of the potential activity of PS II, and the decrease in QP showed a reduction in activity of the PS II reaction center [27]. The decreasing *NPQ* along with the increase of aniline concentration showed that the amount of heat dissipation which was used by antenna pigment descended too. The prolonged aniline stress might affect on Violaxanthin de-Epoxidase, which caused the declined heat dissipation, a process depending on the lutein cycle [24]. Sunflower PS Ⅱ photosynthetic system suffered obvious injury by bright light. The research of Zeming Wang [22] showed that as the concentration of Naphthalene and 1, 2, 4-trichlorobenzene in soil increases, *NPQ* decreased rapidly, and the self-protection mechanism of rice through thermal dissipation had been seriously damaged.

Under normal circumstances, the generation and removal of intracellular free radicals in a dynamic equilibrium state, the low level free radical will not hurt the cells. However, under stress, the accumulation of reactive oxygen species in the plant cells will lead to cell membrane and entire cell damage, and even death of the cell [28]. Adversity induced the plant body to start their own protection mechanism to minimize the damage. Plant SOD, POD and CAT together constitute an antioxidant protective enzyme system, which can remove free radicals such as O_2 -, - OH and so on in the plant under adverse stress, and relieve or reduce the damage degree of plants. Among them, SOD is a plant through the catalytic O_2-conversion to O_2 and H_2O_2, POD will transfer H_2O_2 into O_2 and H_2O, to eliminate the harmful effect of free radicals [29]. The increase of SOD and POD activities in this plant under aniline stress is a mechanism of plant self-protection against stress. The results found through this study showed that the activity of SOD and POD in sunflower increased with the increase of aniline concentration, which indicated that aniline stress could increase the activity of reactive oxygen species and promote the increase of SOD and POD activity. The cells produced a significant toxic effect. This finding is similar to that of Wen Guangjun [30] and Bai Weirong [31], indicating that low concentration of aniline can promote the increase of SOD and POD activity. Sheng found the activity of SOD and POD in sunflower increased first and then decreased with the increase of salt concentration, which indicated that low concentration of salt could obviously promote the activity of SOD and POD in sunflower, while the high concentration salt would inhibit the activity of sunflower SOD and POD [32]. The activity of SOD and POD in this experiment did not decrease with the increase of aniline concentration, which indicated that the 180 mg/L aniline concentration could not inhibit the activity of SOD and POD in sunflower.

In summary, the low concentration of aniline (<120 mg/L) could promote the normal growth of sunflower within a short period of time. When the concentration of aniline gradually increased, the photosynthetic rate of sunflower decreased. However, when the duration of stress reaches a certain level, aniline will have a significant toxic effect on sunflower, seriously affecting the normal growth of sunflower.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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